Reactivity of Alkanals towards Malondialdehyde (MDA) and the Effect of Alkanals on MDA Determination with a Thiobarbituric Acid Test

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The reactivity of formaldehyde (FA), acetaldehyde (AA) and propanal (PA) towards malondialdehyde (MDA) under physiological conditions was investigated. These alkanals revealed a reactivity towards MDA. Especially, FA showed high reactivity; for instance, a mixture of MDA (1 mM) and FA (6 mM) incubated for 4 h, or a mixture of MDA (1 mM) and FA (1 mM) incubated for 24 h, resulted in almost a 100% decrease of MDA. The amount of free MDA decreased as the alkanal concentration increased and in the reaction of MDA with AA or PA, the loss of MDA was in approximate agreement with the yield of 2,4-dihydroxy methylene-3-alkylglutaraldehyde (1b or 1c). In the reaction of MDA with FA, the yield of 1a decreased with an excess amount of FA. In a thiobarbituric acid (TBA) test, I gave a positive reaction. Compounds 1b and 1c, which contain 2 molecules of MDA, liberated 1 molecule of MDA during the TBA reaction. The results showed that the MDA recovery of 1b and 1c with the TBA test was 50%. In the case of 1a, MDA recovery was 32%. In the MDA/p-nitrophenylhydrazine (NPH)-HPLC method, the MDA recovery of 1a, 1b and 1c ranged from 3 to 7%.

Keywords malondialdehyde; thiobarbituric acid test; formaldehyde; acetaldehyde; propanal

Malondialdehyde (MDA) is formed from the peroxidation of polyunsaturated fatty acids, from the degradation of deoxyribose by a hydroxyl radical and as a by-product of prostaglandin biosynthesis. MDA has been implicated in aging, mutagenesis and carcinogenesis. Since MDA has long been used as an indicator of the extent of oxidative rancidity that has occurred in foods and of the extent of lipid peroxidation occurring in various in vivo situations such as aging or degenerative diseases, various methods, such as the thiobarbituric acid (TBA) test, the direct determination method by high-performance liquid chromatography (HPLC), MDA/TBA-HPLC methods, MDA/phenylhydrazine-HPLC methods and gas chromatography (GC) methods, have been developed to estimate MDA in biological samples. Therefore, it is important to investigate the stability of MDA and the reactivity of MDA towards biological substances under physiological conditions.

Sanchez et al. reported that 2,4-dihydroxy methylene-3-alkylglutaraldehyde (1, Chart 1) was produced by the reaction of MDA with alkanals. On the other hand, it is known that alkanals such as formaldehyde (FA), acetaldehyde (AA), propanal (PA) and hexanal are produced along with MDA as the end products of lipid peroxidation.

In this study, the reactions of alkanals with MDA under physiological conditions were investigated. This paper describes the reactivity of alkanals (FA, AA and PA) towards MDA under physiological conditions and the relation between the loss of MDA and the yield of 1.

Moreover, the TBA reaction with 1 was investigated. The most widely employed method for the determination of MDA is the TBA test, in which one molecule of MDA reacts with 2 molecules of TBA, resulting in the production of a red pigment having an absorption maximum at 532 nm. It has been shown that bound MDA, such as the 1:1 Schiff's base of MDA with a primary amine and an MDA–guanine adduct, also react with TBA to give a red pigment with an absorption maximum at 532 nm. Therefore, in this study, the TBA test with 1 and the MDA recovery of the reaction mixture of an alkanal and MDA with a TBA test were also investigated.

Materials and Methods

Apparatus Absorbances were measured with a Shimadzu UV-150-02. HPLC was performed with a Shimadzu LC-6A equipped with a Shimadzu SPD-6A UV absorption detector.

Materials Sodium malondialdehyde (MDA–Na), 2,4-dihydroxy methylene glutaraldehyde (1a), 2,4-dihydroxy methane-3-methyl glutaraldehyde (1b), and 2,4-dihydroxy methane-3-ethyl glutaraldehyde (1c) were purchased from Merck Co., Ltd.; TBA, p-nitrophenylhydrazine (NPH) hydrochloride and PA were from Tokyo Kasei Co., Ltd.; FA and tetra decyltrimethylammonium bromide were from Wako Chemical Industries. TBA was used after being recrystallized from water. The concentration of the FA solution was determined according to the method described in the literature.

Effect of Alkanal Concentration on the Loss of MDA A mixture of MDA–Na (1 mM) and FA, AA or PA (0–10 mM) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37 °C for 4 h. Aliquots (1 mL) were removed, diluted with water to 10 mL, and 10 μL of the solution was injected into HPLC to determine the remaining MDA. HPLC analysis was performed according to the procedure of Behrens and Madere. The procedure was as follows: a 4.6 × 250 mm octadecyl silica (ODS) column (Capcell pack SG-120, Shiseido) was eluted at 1.0 mL/min with 0.03 M phosphate buffer (pH 6.5) containing 30% ethanol and 1 mM tetradecyltrimethylammonium bromide. The absorbance was measured at 267 nm.

Formation of A mixture of MDA–Na (1 mM) and FA, AA or PA (0–10 mM) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37 °C for 4 h. Aliquots (1 mL) were removed, diluted with water to 10 mL and 10 μL of the solution was injected into HPLC for the determination of 1a, 1b or 1c. HPLC analysis was performed on a 4.6 × 250 mm ODS column (Capcell pack SG-120) with a mobile phase of 0.01 M phosphate buffer (pH 7.0) containing 10% methanol at a flow rate of 0.8 mL/min (for the determination of 1a) or with a mobile phase of 0.01 M phosphate buffer (pH 7.0) containing 25% methanol at a flow rate of 1 mL/min (for the determination of 1b and 1c). The UV absorbance at 250 nm was integrated and the concentration of 1a, 1b or 1c was determined using a standard curve.

TBA Test The TBA assay was performed according to Estebauer and
Cheeseman\textsuperscript{20} with slight modifications. A mixture of 1 ml of 10 \( \mu \)M MDA, 1a, 1b or 1c solution, 2 ml of 10\% (w/v) trichloroacetic acid (TCA) solution and 1 ml of 0.67\% (w/v) TBA solution was heated at 95—100\degree C for 30 min. After cooling, the absorbance at 532 nm was measured. The determination of the MDA—TBA adduct by HPLC was performed according to Squires\textsuperscript{20} with slight modifications. A mixture of 1 ml of MDA or 1b solution (0.2 mm), 2 ml of 5\% (w/v) TCA solution and 1 ml of 0.67\% (w/v) TBA solution was heated at 95—100\degree C for 30 min. After cooling, the mixture was diluted with 0.1 M phosphate buffer (pH 8.0) to 20 ml, then 10 \( \mu l \) of the solution was injected into HPLC. HPLC analysis was performed on a 4.6 \times 250 mm ODS column (Ultron C\textsubscript{18}, Shinwaakakou Co., Ltd.) with a mobile phase of 0.01 M phosphate buffer (pH 8.0) containing 15\% acetonitrile and 0.65\% tetrahydrofuran at a flow rate of 1 ml/min. The absorbance was measured at 532 nm.

**MDA/NPH-HPLC Method** The MDA/NPH-HPLC method was performed according to Kawai et al.\textsuperscript{28} A mixture of 1 ml of MDA, 1a, 1b or 1c solution (0.1 mm), 0.6 ml of 1 M acetate buffer (pH 3.8) and 0.2 ml of NPH-HCl ethanol solution (0.05\%) was allowed to stand at room temperature for 1 h, and 10 \( \mu l \) of the mixture was injected into HPLC. HPLC analysis was performed on a 4.6 \times 250 mm ODS column (Capcell pack SG-120) with a mobile phase of 0.01 M sodium dihydrogen phosphate containing 30\% acetonitrile and 10\% isopropanol at a flow rate of 1 ml/min. The absorbance was measured at 315 nm.

**Results**

**Effect of Alkanal Concentration on the Loss of MDA** The relationship between alkanal concentration and the loss of MDA under physiological conditions was examined. Among alkanals which can be formed during lipid peroxidation, FA, AA and PA were used in the experiments since these alkanals were easily soluble in water. A mixture of MDA (1 mm) and alkanal (0—10 mm) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37\degree C for 4 h, and the MDA which remained was determined by the HPLC method. As shown in Fig. 1, these alkanals showed reactivity towards MDA, and the amount of free MDA decreased as the alkanal concentration increased. Especially, FA strongly reduced the amount of free MDA, which was fully consumed at an FA concentration of 6 mm.

**Time Course of the Loss of MDA** Since alkanals are produced in larger amounts than MDA during lipid peroxidation,\textsuperscript{5,37} the time course experiments on the loss of MDA were carried out in an excess amount of alkanal. Mixtures of MDA (1 mm) and FA (1 or 3 mm), AA (3 or 10 mm) or PA (3 or 10 mm) in 0.1 M phosphate buffer (pH 7.4) were incubated at 37\degree C. The results are shown in Fig. 2. In all cases, the amount of free MDA gradually decreased as the reaction time increased, while MDA alone was stable even after 24 h. In the reaction of MDA with AA and PA, free MDA remained after 24 h even at alkanal concentration of 10 mm, whereas in the reaction with FA, free MDA was consumed within 8 h at an FA concentration of 3 mm or within 24 h at an FA concentration of 1 mm.

**Formation of 1** It has been reported that 1 was isolated from the reaction of MDA with an alkanal.\textsuperscript{33,34} Thus, the author investigated whether 1a, 1b and 1c were also formed under physiological conditions. Figure 3 shows the relationship between alkanal concentration and the yields of 1a, 1b and 1c in the reactions of MDA (1 mm) with alkanals (0—10 mm) at pH 7.4. In the cases of AA and PA, the yields of 1b and 1c increased as the alkanal concentration increased. From Figs. 1 and 3, the yields of 1b and 1c were in approximate agreement with the loss of MDA caused by AA and PA. In the case of FA, the yield of 1a increased as the FA concentration increased up to 2 mm, then decreased with a further increase in FA concentration. The decrease of 1a with an excess amount of FA seemed to be caused by further reaction of 1a with FA, since FA is a highly reactive aldehyde. Sanchez et al.\textsuperscript{34} reported that 1a and 1b were obtained in 10\% and 60\% yields, respectively, by the reaction of 2 mol of MDA and 1 mol of FA or AA. However, under physiological conditions, 1a was formed in a higher yield (58\%), and an excess amount of AA was required to give 1b in high yield.

**Time Course of the Formation of 1** The time course of the formation of 1a, 1b and 1c in the reaction of MDA (1 mm) with FA (1 or 3 mm), AA (3 or 10 mm) or PA (3 or 10 mm) at pH 7.4 was charted (Fig. 4). At an alkanal concentration of 3 mm, the yield of 1b and 1c gradually increased as the reaction time increased, whereas the yield

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Fig. 1. Effect of Alkanal Concentration on the Loss of MDA

A mixture of MDA (1 mm) and alkanal (0—10 mm) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37\degree C for 4 h. O, FA; ●, AA; △, PA.

Fig. 2. Time Course of the Loss of MDA

Mixtures of MDA (1 mm) and FA (O, 1 mm; ●, 3 mm; △, MDA alone) (A); MDA (1 mm) and AA (O, 3 mm; ●, 10 mm) (B) or MDA (1 mm) and PA (O, 3 mm; ●, 10 mm) (C) in 0.1 M phosphate buffer (pH 7.4) were incubated at 37\degree C.
of 1a reached a maximum after 5 h and then decreased with increasing reaction time. After 24 h, the amount of 1a was reduced to 1%. At the highest alkanal concentration (10 mM), the yields of 1b and 1c also reached a maximum after 5 h (1b) and 8 h (1c), respectively, then gradually decreased with increasing reaction time.

**TBA Test** The MDA recovery of 1a, 1b and 1c on the MDA determination by TBA test was investigated. The color development in the TBA test requires exposure of the reactant to high heat (95—100 °C), and heating time periods ranging from 15 min to over 1 h were employed. Figure 5 shows the effect of heating time on color development in the TBA tests of 1a, 1b and 1c. These compounds gave a positive TBA reaction. The color development of the TBA reaction with these compounds reached a maximum after 30 min, whereas that with MDA reached a maximum after 15 min. It has been reported that the TBA test of biological materials produces pigments which show absorbance at 532 nm, except for the MDA–TBA adduct. In order to investigate whether the pigment produced by the TBA reaction with 1 was identical to that of the MDA–TBA adduct, the HPLC procedure which had been employed only for the determination of the MDA–TBA adduct in the TBA test was now carried out. Figure 6 shows the HPLC elution profiles of the reaction mixture of TBA with MDA and that of TBA with 1b. The HPLC elution profiles indicated that the pigment produced by the TBA reaction with 1b was identical with that of the MDA–TBA adduct. From the above results, 1b and 1c, which contain 2 molecules of MDA, liberated 1 molecule of MDA during the TBA reaction since the absorbances of 10 μM of 1b and 1c were almost the same as that of 10 μM MDA in the TBA reaction (Fig. 5). This shows that the MDA recovery of 1b and 1c with the TBA test was about 50%. In the case of 1a, MDA recovery was only 32%. Since MDA reacted with an alkanal to give 1, a mixture of MDA and an alkanal incubated for 24 h gave low MDA recovery according to the TBA test (Table I). Especially, a mixture of MDA (1 mM) and FA (3 mM) incubated for 24 h gave an extremely low recovery of MDA (5%) with the TBA test. This shows that the reaction of MDA with an excess amount of FA gave substances which did not liberate MDA during the TBA reaction. The structures of the substances could not be identified in this study. On the other hand, a mixture of MDA and an alkanal without incubation gave a quantitative MDA recovery (Table I). This shows that these alkanals did not react with MDA during the TBA reaction. Kosugi et al. reported that a red pigment having an absorption maximum at 532 nm was produced when a mixture of an alkanal and alkenal and TBA in acetic acid solution was heated at 100 °C over 2 h. However, under the reaction conditions employed in this experiment, these alkanals did not produce the red pigment during a TBA reaction (Table I).

**MDA/NPH-HPLC Method** Several researchers have developed methods for the determination of free MDA by using HPLC or GC after derivation with hydrzone compounds such as NPH and N-methylhydrazine. Of these methods, the MDA/NPH-HPLC method was examined.

**Table I. TBA Test of the Reaction Mixture of MDA and Alkanal**

<table>
<thead>
<tr>
<th>TBA reactants</th>
<th>Ratio of absorbance in TBA test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (1 mM)</td>
<td>100</td>
</tr>
<tr>
<td>MDA (1 mM) + FA (1 mM)</td>
<td>25</td>
</tr>
<tr>
<td>MDA (1 mM) + FA (3 mM)</td>
<td>5</td>
</tr>
<tr>
<td>MDA (1 mM) + AA (10 mM)</td>
<td>48</td>
</tr>
<tr>
<td>MDA (1 mM) + PA (10 mM)</td>
<td>61</td>
</tr>
<tr>
<td>MDA (1 mM) + FA (10 mM)</td>
<td>100</td>
</tr>
<tr>
<td>MDA (1 mM) + AA (10 mM)</td>
<td>100</td>
</tr>
<tr>
<td>MDA (1 mM) + PA (10 mM)</td>
<td>100</td>
</tr>
<tr>
<td>FA (10 or 100 mM)</td>
<td>0</td>
</tr>
<tr>
<td>AA (10 or 100 mM)</td>
<td>0</td>
</tr>
<tr>
<td>PA (10 or 100 mM)</td>
<td>0</td>
</tr>
</tbody>
</table>

a) A mixture of MDA and alkanal in 0.1 M phosphate buffer (pH 7.4) was incubated at 37 °C. The reaction mixture was diluted 100-fold with water, and 1 ml of the solution was subjected to the TBA test. b) The reaction mixture was incubated for 24 h. c) The reaction mixture was subjected to the TBA test without incubation.
forms, the structures of which are believed to be adducts with primary amino groups of biomolecules.\textsuperscript{52,53} Nair \textit{et al.}\textsuperscript{54} demonstrated that MDA showed high reactivity towards primary amines, such as amino acid methyl esters, to give Schiff bases under acidic conditions. Table III shows the reactivities of alkanals and \textit{n}-hexylamine (HA) towards MDA under physiological conditions. A mixture of MDA (1 mM) and an alkanal (3 mM) or HA (3 mM) in a phosphate buffer (pH 7.4) was incubated for 4 h, then the remaining free MDA was determined by the HPLC method. The results showed that these alkanals had higher reactivities towards MDA than did HA.

It has been reported that when MDA was added to urine, free MDA gradually decreased.\textsuperscript{18,32} Bagchi \textit{et al.}\textsuperscript{55,56} demonstrated that FA was present in urine in a relatively large amount, and an increase of FA was observed in response to lipid peroxidation. Therefore, it can be assumed that a decrease in free MDA in biological samples such as urine may be due in part to the formation of an adduct of MDA and FA.

Although the TBA test has been described as one of the most useful methods, it has occasionally shown poor correlation with other oxidative parameters. For instance, the TBA values decreased after a certain incubation period for some samples, such as freeze-dried meat\textsuperscript{57} and microsomes,\textsuperscript{58} when peroxidation was still progressing. Buttkus and Bose\textsuperscript{49} reported that the poor correlation between TBA values and other oxidative parameters was due to the formation of adducts of MDA and sulphydryl groups of protein, since the MDA-cystein adduct, in contrast to MDA-primary amine adduct, gave low MDA recovery with the TBA test. However, Shin \textit{et al.}\textsuperscript{59} noted that there is little reactivity of MDA toward sulphydryl groups since the loss of the sulphydryl of \textit{N}-acetylcystein in the presence of an excess amount of MDA was not significantly greater than in the absence of MDA. The author also obtained the same result. When a mixture of MDA (1 mM) and \textit{N}-acetylcystein (10 mM) was incubated for 4 h, the amount of MDA did not decrease (Table III). Therefore, the decrease in TBA value may be due to the reaction of MDA with alkanals, rather than to its reaction with a sulphydryl group of protein, since the MDA solution incubated in the presence of an alkanal gave low MDA recovery with the TBA test (Table I).

From these results, it was suggested that alkanals which are produced along with MDA during lipid peroxidation can react with MDA and may result in a low recovery of MDA as determined in biological samples using the direct HPLC method, a TBA test and the MDA/NPH-HPLC method. In addition, the reaction can result in different rates

**Table III. Reactivity of Alkanal, \textit{N}-Acetylcysteine and HA towards MDA**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDA remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (1 mM) + FA (3 mM)</td>
<td>13</td>
</tr>
<tr>
<td>MDA (1 mM) + AA (3 mM)</td>
<td>71</td>
</tr>
<tr>
<td>MDA (1 mM) + PA (3 mM)</td>
<td>77</td>
</tr>
<tr>
<td>MDA (1 mM) + HA (3 mM)</td>
<td>96</td>
</tr>
<tr>
<td>MDA (1 mM) + \textit{N}-acetylcysteine (10 mM)</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} A reaction mixture in 0.1 M phosphate buffer (pH 7.4) was incubated at 37°C for 4 h.

in order to see whether I was determined to be MDA. As shown in Table II, MDA recovery was only 3—7%. Accordingly, by using the MDA/NPH-HPLC method, in contrast to the TBA test, I was hardly determined to be MDA. This indicated that MDA was not significantly liberated from I, since the MDA/NPH-HPLC method was performed under mild conditions.

**Table II. MDA Recovery of I Using the MDA/NPH-HPLC Method**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDA recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>3</td>
</tr>
<tr>
<td>1b</td>
<td>4</td>
</tr>
<tr>
<td>1c</td>
<td>7</td>
</tr>
</tbody>
</table>

**Discussion**

MDA, a decomposition product of lipid peroxide, is used as an indicator of oxidative damage to cells and tissues. It is well established that during lipid peroxidation, MDA is formed together with other aldehydes such as alkanals.\textsuperscript{5,35—37} Therefore, it is important to investigate the reactivity of alkanals towards MDA under physiological conditions. As shown in Fig. 1, alkanals such as FA, AA and PA showed reactivity towards MDA. Especially, FA showed high reactivity.

It has been reported that MDA produced by lipid peroxidation in biological samples is converted to bound
of MDA recovery depending on whether the direct HPLC method or TBA test was used.

References
49) H. Kosugi, K. Kikugawa, Lipids, 21, 537 (1986).